



**REPLY UNDER 37 C.F.R. 1.116 – EXPEDITED PROCEDURE
TECHNOLOGY CENTER 1633**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Inventors	: Véronique Trochon	Docket No.: 1002-04
	: He Lu	
	: Claudine Soria	Confirmation No.: 9953
Title	: METHOD OF INHIBITING	
	: ANGIOGENESIS OR	
	: INVASION OR FORMATION	
	: OF METASTASES	Dated: May 8, 2007

DECLARATION OF VÉRONIQUE TROCHON-JOSEPH

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Commissioner for Patents
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Sir:

I, Véronique Trochon-Joseph, declare that I reside at 83, rue Didot, 75014 Paris, France. I attended the University of Rouen and received a PhD degree in Pharmacology Specialization in 1998. Since 2001, I have been employed by Bioalliancepharma Company as a Project Manager where I developed the anti-angiogenic and anti-invasive metargidin recombinant disintegrin peptide project. I have published 11 articles in international scientific papers.

I am one of the inventors named in the above-identified U.S. Patent Application, that I am thoroughly familiar with the above referenced patent application and the subject matter described and claimed therein;

In an effort to demonstrate the fact that our original disclosure complies with 35 U.S.C. §112, we performed a number of experiments based on the original disclosure in our Specification. Those experiments and the results that we obtained are reported below.

AMEP plasmid results

Plasmid systems

Two different plasmid systems coding for the RDD gene were constructed : the Tet-inducible pBi system, and the constitutive pVAX plasmid.

1) The RDD transgene was expressed under the control of a tetracycline-inducible Tet-On CMV promoter. The tetracycline system used is composed of three ampicillin resistant plasmids:

- The first plasmid (pBi-RDD) contains a CMV (cytomegalovirus) promoter with a tetracycline-responsive element (TRE) to drive the expression of the *AMEP* gene. The RDD gene under the control of the murine urokinase secretion signal was inserted between the P_{MNCMV1} promoter and the β -globin polyA
- The second plasmid, pTet-On, expresses a strong transcriptional activator, rtTA.
- The third plasmid, pTet-tTS, encodes a powerful transcriptional silencer (tTS) that, in the absence of doxycycline, binds to the TRE sequence and blocks expression of the gene of interest.

2) pVAX1 (Invitrogen, ref V260-20) is a 3.0 kb plasmid vector designed for use in development of DNA vaccines. Features of the vector allow high-copy number replication in *E. coli* and high-level transient expression of the protein of interest in most mammalian cells. The vector contains the following elements:

- Human CMV promoter
- Bovine growth hormone (bGH) polyadenylation signal
- Kanamycine resistance gene for selection in *E. coli*

The RDD cassette, containing the human *AMEP* gene under the control of the murine urokinase secretion signal, was excised from the pBi-RDD and cloned in pVAX1 between the CMV and the BGH polyA, to generate the pVAX-RDD.

Pre-established murine B16F10 melanoma model

We injected 10^6 B16F10 murine melanoma cells in subcutaneous route onto the dorsa of C57Bl/6 mice. Tumors were intratumorally injected with

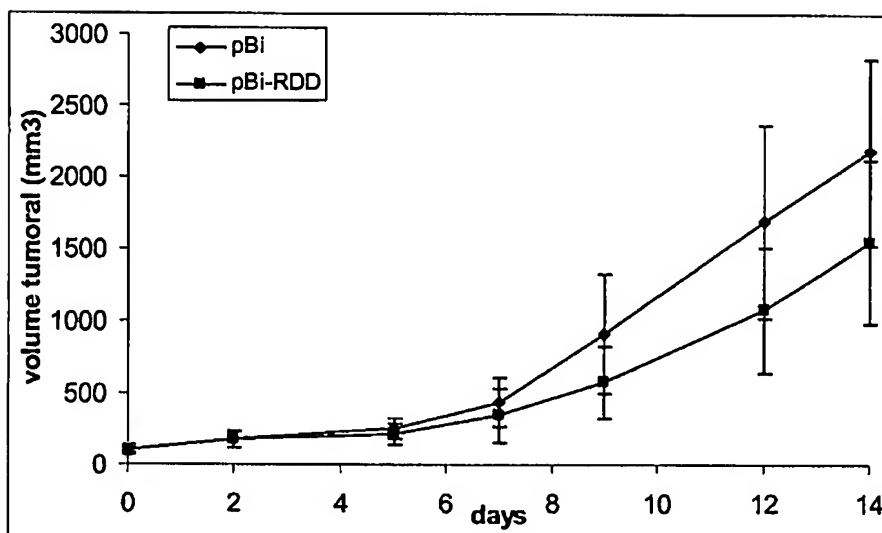
- 20 μ g of pBi control plasmid or pBi-RDD plasmid, mixed with 20 μ g of pTet-On and 10 μ g of pTet-tTS plasmids (in 50 μ l NaCl 0,9%).
- Or 50 μ g of pVAX control plasmid or 50 μ g of pVAX-RDD plasmid in 50 μ l

Conductive gel was applied on the tumor. The injection was immediately followed by electric pulses application by use of two stainless steel plate electrodes placed 5 mm apart on the tumor, according to the following protocol:

- HV = 1500 V/cm, 100 μ s, 1 Hz, 1 pulse
- pause = 1 000 ms
- LV = 140 V/cm, 400 ms, 1 pulse

Day 0= treatment day.

Tumor size was monitored by measuring two perpendicular diameters with a digital caliper. Tumor volume was calculated according to the formula: $(\text{length} + \text{width}/2)^3 \times \pi/6$.

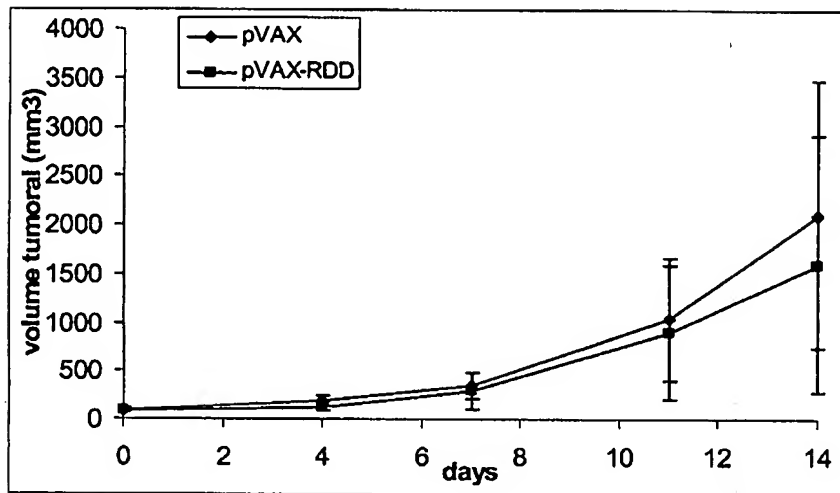


Summary: tumor volume in mm3

days	0	2	5	7	9	12	14
pBi	104.45	180.91	252.52	436.90	913.77	1692.89	2179.24
SD pBi	14.90	54.87	73.80	168.60	415.83	673.10	651.91
pBi-RDD	108.33	180.74	217.26	351.60	582.80	1083.70	1550.86
SD RDD	37.51	60.62	72.53	189.19	252.35	431.57	565.86
% inhibition		0.1	14.0	19.5	36.2	36.0	28.8
SigmaStat Student p=	0.764	0.995	0.295	0.301	0.045	0.03	0.05
					Mann&Whitney	0.055	0.092

We observed a tumor growth inhibition in the murine melanoma B16F10 pre-established model. This inhibition (at least 30%) was significant from day 9 to day 14.

In this model, the pVAX-RDD plasmid was inefficient to significantly inhibit the B16F10 tumor growth.



B16F10 metastatic model

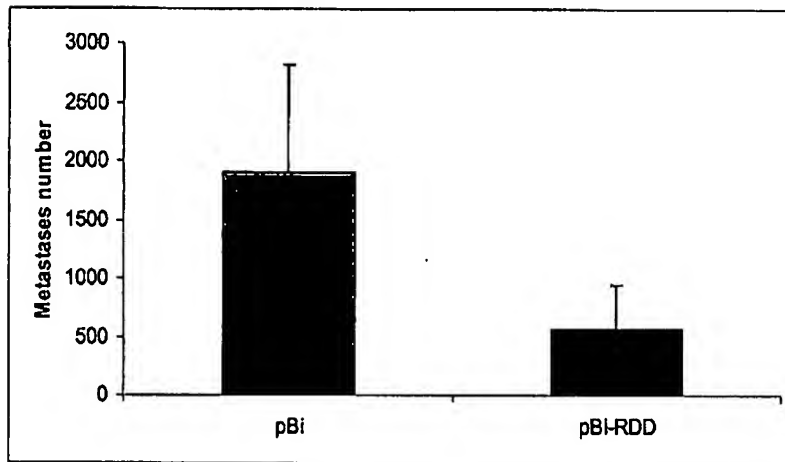
Day 0: Injection of

- 20µg of pBi control plasmid or pBi-RDD plasmid, mixed with 20µg of pTet-On and 10 µg of pTet-tTS plasmids (in 50µl NaCl 0,9%).
- Or 50µg of pVAX control plasmid or 50µg of pVAX-RDD plasmid in 50µl into both *Tibialis cranialis* muscles of C57Bl/6 mice, immediately followed by electrotransfer with the Cliniporator device

electrotransfer parameters : HV = 700 V/cm, 100µs, 1hz, 1 pulse
 pause = 1000 ms
 LV = 100 V/cm, 400 ms, 1 pulse

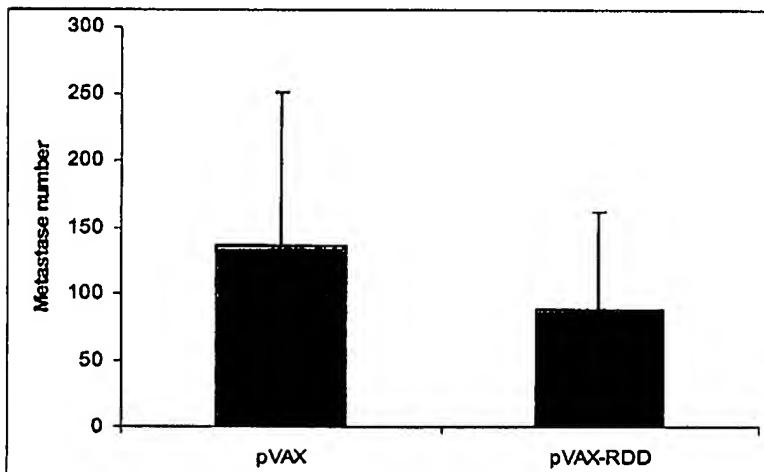
Day 3: Injection of 400 000 B16F10 cells (in 100µl) by IV route into the retro-orbital sinus.

Day 10 : Mice were sacrificed and lung metastatic loci were counted



	mean	SD	% inhib.	Student SigmaStat
pBi	1906.50	911.63		
pBi-RDD	561.75	378.01	70.5	0.008

We observed 70% of inhibition of the metastatic development with the pBi plasmid.



	mean	SD	% inhib.	
pVAX	136.67	114.23		
pVAX-RDD	88.50	72.62	35.2	p=0,32 Student SigmaStat

We observed 35% of inhibition of the metastatic development with the pVAX plasmid.

Pre-established human C9 melanoma model

We injected 1×10^6 C9 cells in subcutaneous route onto the dorsa of Swiss Nude mice. When mean tumor volume reached 50 mm^3 , tumors were intratumorally injected with:

- $20 \mu\text{g}$ of pBi control plasmid or pBi-RDD plasmid, mixed with $20 \mu\text{g}$ of pTet-On and $10 \mu\text{g}$ of pTet-tTS plasmids (in $50 \mu\text{l}$ NaCl 0,9%).
- Or $50 \mu\text{g}$ of pVAX control plasmid or $50 \mu\text{g}$ of pVAX-RDD plasmid in $50 \mu\text{l}$.

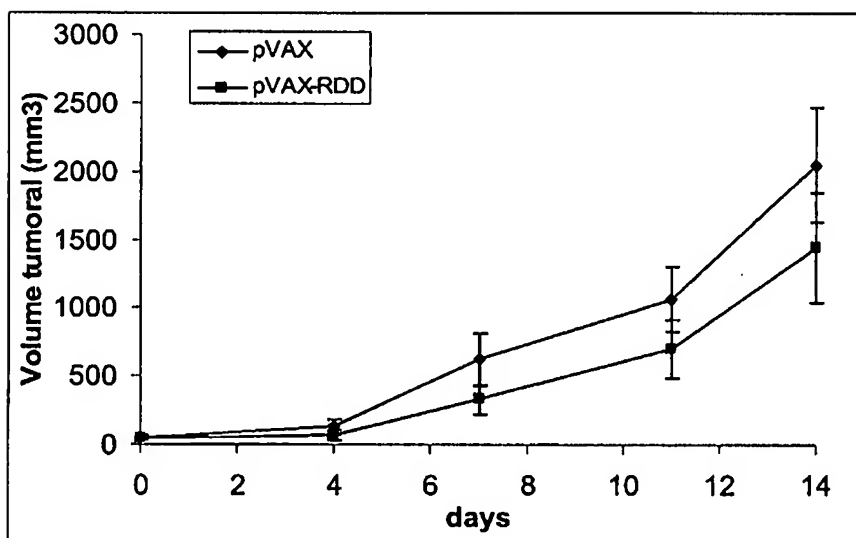
Conductive gel was applied on the tumor. The injection was immediately followed by electric pulses application by use of two stainless steel plate electrodes placed 5 mm apart on the tumor, according to the following protocol:

- HV = 1500 V/cm , $100 \mu\text{s}$, 1 Hz, 1 pulse
- pause = 1 000 ms
- LV = 140 V/cm , 400 ms, 1 pulse

Day 0 = treatment day.

Tumor size was monitored by measuring two perpendicular diameters with a digital caliper.

Tumor volume was calculated according to the formula: $(\text{length} + \text{width}/2)^3 \times \pi/6$.



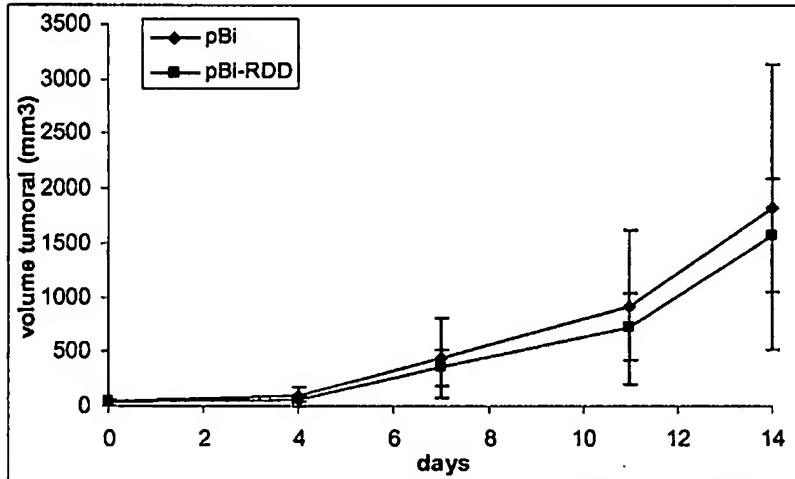
Summary: tumor volume in mm³

days	0	4	7	11	14
pVAX	50.64	131.87	621.69	1066.99	2050.98
SD pVAX	18.17	50.53	195.15	235.19	425.19
pVAX-RDD	51.99	75.77	335.19	701.14	1443.22
SD pVAX-RDD	20.03	34.44	103.72	215.00	404.03
% inhibition		42.5	46.1	34.3	29.6
SigmaStat Student p=	0.900	0.029	0.004	0.010	0.018

We observed a tumor growth inhibition in the human melanoma C9 pre-established model. This

inhibition (at least 30%) was significant from day 4 to day 14. The maximum of inhibition (46%) was obtained at day 7 post-treatment.

The inducible pBi-RDD plasmid was inefficient to significantly inhibit the C9 tumor growth.



The undersigned declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and thus such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 26, 2007

Véronique Trepoignon-Josset Co-inventor